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Intact Killifish *(Fundulus heteroclitus)* as a Tool for Medically Oriented Study of Marine Neurotoxins[1]

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(Plates I-II; Text-figure 1)

Using *Fundulus heteroclitus* (killifish), an abundant teleost fish in which aggregation-dispersion of melanophore pigment is rapid and predominantly nerve regulated, it was observed that systemic administration of tetrodotoxin or saxitoxin (purified shellfish poison or derived from *Gonyaulax catenella*) caused sectoral darkening, interpreted as following the previously reported pattern of peripheral pigment-motor unervation, indicating peripheral, central nervous system effects. The test can be used as a sensitive and specific assay for these toxins. Concurrent quatitative and qualitative analysis as well as screening for neuroactive toxins is possible using intraperitoneal or intragas-bladder injection of fish weighing 6-7 gm.(adv.). A consideration of our results and those of others indicates that *Fundulus herteroclitus* lacks the long postulated melanin dispersing nerve.

INTRODUCTION

THIS STUDY arose from work by Burke et al. (1960) and Ruggieri et al. (1962) with axenic cultures of *Gonyaulax* species and other dinoflagellate toxins, and from our discovery of a specific pigmentary response in *Fundulus* upon systemic administration of these toxins or tetrodotoxin. Using this response, we developed a sensitive and specific assay system for tetrodotoxin and saxitoxin. To do this, it was first necessary to further define *Fundulus* (1) by observing the effect of physical factors, and (2) through administration of drugs having known mechanisms of action. These were correlated with previously known facts regarding *Fundulus* and with subsequently published findings regarding the mechanism of action of saxitoxin and tetrodotoxin.

Relevant previously known facts are as follows:

1. *Fundulus heteroclitus* belong to that group of teleosts whose color changes are primarily under nervous control (Fingerman, 1963) as exerted through their melanin-aggregating (blanching or paling) sympathetic nerves (Wyman, 1924a) (Text-fig. 1).

2. There is no *direct* evidence that any opposing melanin-dispersing pigmentomotor nerves exist in *Fundulus* (Pye, 1964a).

3. A pituitary melanin-dispersing hormone (M.D.H.) must play a part in opposing sympathetic nerve effects, for although melanophores of intact *Fundulus* are nonresponsive to M.D.H. (Pickford & Kosto, 1957), denervated tail melanophones cannot expand to a black background in the absence of M.D.H. (Abramowitz, 1940; Pickford & Kosto, 1957).

4. *Fundulus* pituitary extracts contain, and their melanophores respond to, a melanin aggregating hormone (M.A.H.) (Pickford & Atz, 1957). Pineal substance aggregates melanophores of embryonic and larval *Fundulus* but not adult *Fundulus* (Wyman, 1924b) (Lerner's melanin aggregating hormone, melatonin, has since been isolated from the pineal gland [Lerner et al., 1958]).

5. *Fundulus* melanophores are neural crest derivatives and react directly to many neurotropic agents, as other investigators including Scheline (1963), Wyman (1924a), and Abbott (1968) have shown by work on isolated scales; however, many of these reactions are probably mediated by release of transmitter substance

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from presynaptic membrane, as Fujii & Novales (1968) found for tetrodotoxin.

6. It has been stated by von Frisch (1911) and quoted by Brown (1957), Pye (1964a), Wyman (1924a), and others that the melanin-aggregating nerves of *Phoxinus phoxinus L*, a *Fundulus*-like teleost, emerge from the spinal canal at the 15th vertebra, bifurcating anteriad and posteriad (Text-fig. 1).

Tetrodotoxin (Narahashi et al., 1964) and saxitoxin (Kao & Nishiyama, 1965) were subsequently reported to interrupt neuronal function by blocking the rapid passive flux of sodium into nerve cells without affecting the sodium pump or the inward or outward shifts of potassium (Kao, 1966). No other agents are known to work precisely this way. In contrast, commonly employed pharmacological agents, such as local anesthetics and barbiturates, block both sodium and potassium ion passive flux (Frank & Sanders, 1963; Frank & Pinski, 1966; Narahashi et al., 1964; Narahashi et al., 1967). For reasons discussed in depth elsewhere (Down & McLaughlin, 1969), we believe the unique response of *Fundulus* to saxitoxin and tetrodotoxin reflects this difference, as well as the peculiarities of *Fundulus* melanophore innervation.

Materials and Methods

Toxin sources. Dinoflagellate toxins were obtained by growing *Gonyaulax catenella* and *G. tamarensis* in axenic culture. Cells were harvested, extracted, and the poison purified using techniques previously developed by Burke et al. (1960). Samples of purified shellfish poison were obtained from Edward J. Schantz, U.S.A. Chemical Corps Biological Laboratories, Ft. Detrick, Maryland. This shellfish toxin had been prepared as previously reported (Shantz, 1960). Although the dinoflagellate toxin has not been shown to be chemically identical to the shellfish toxin, for brevity these toxins will be called "saxitoxin" (Schuett & Rapoport, 1962), as no pharmacological distinctions have been observed previously or in this work. Crystalline tetrodotoxin prepared by Sankyo Co. was obtained from Dr. C. Y. Kao of the Downstate Medical Center, Brooklyn, New York, on May 12, 1964, and used within that month.

Potency standardization of toxins. One ml volumes of diluted saxitoxin were injected intraperitoneally (I.P.) into 20.7 — 27.1 gram white mice, and the concentration for an average 15-minute death time (1 Mouse unit, or M.U.) determined. Solutions were periodically restandardized with mice to eliminate error from loss of potency occurring over the seven month period of use. Because fish used were smaller than mice, volumes of 0.1 ml were almost always used for fish injection. Tetrodotoxin was standardized using the same technique (0.2 ug was found equal to 1 M.U.).

Saxitoxin; sodium concentration. Two samples of saxitoxin, one acid extracted, the other further purified and concentrated by passage through an ion-exchange column, were assayed for sodium and potassium content by the kidney-and-electrolyte laboratory of the Seton Hall College of Medicine and Dentistry. Except for 43 meq/L sodium in the acid extracted sample, these were present in negligible concentrations.

Preparation of solutions. Solutions were in water (a few in saline) and prepared with HCl or NaOH only if necessary to effect solution, then adjusted to pH 7. Fresh solutions were prepared daily, usually within two hours of use. No aseptic precautions were taken in preparing or injecting solutions.

Test animal. Killifish were trapped from October 1963 through May 1964 in estuaries and tidal pools of Cape May County, New Jersey, where water temperatures as low as −1.5C° were recorded. Occasionally, when this source of supply was unavailable, fish were obtained from the Osborn Laboratories of Marine Sciences, New York Aquarium, Brooklyn, New York. Transported in polyethylene bags, fish showed hypoxia-induced darkening only when shipments were delayed, and such fish were used only for experiments on hypoxia damage. *Fundulus* were maintained in aerated sea water aquaria, kept on a neutral-shaded background in an air conditioned room (24-28°C). Unless otherwise stated, light-adapted fish over a light background were used. All experiments were performed at normal room illumination.

Fish were generally used within 48 hours of collection. They were placed on a light or dark background for one-half hour before injection, and after treatment were placed in culture dishes over light (white porcelain pans) or dark (wet carbon paper) backgrounds.

Injection of test animals (13 mm long, 27 gauge needles). With practice, capture and restraint of fish in a bare, moistened hand took no longer than 10 seconds. For preliminary or quantitative work, the injection method of choice proved to be intra-gas-bladder (i.g.b.). Subcutaneous and intramuscular injections gave variable results, probably reflecting chance injection near nerves or blood vessels. Intraperitoneal (i.p.) injections were easier but lacked the advantage of i.g.b. injections of entry confirmation by gas aspiration. Aspiration attempted before i.p. injection ruled out gas bladder or blood vessel entrance. To avoid damaging major vessels

during i.g.b. injection, the needle was not permitted to contact the vertebral bodies. Occasionally, i.g.b. injection caused vascular damage visible through the body wall, and fish so effected were discarded.

A minimum of three fish were injected for each concentration of each solution tested.

Timing of melanophore responses, melanophore recoveries, and death times. Times of earliest visible melanophore changes were recorded. Death times represent the irreversible cessation of visible opercular movements.

Weighing Fundulus. All fish were drained of excess water and weighed immediately.

Photography. Kodak Panatomic 35-mm film was exposed by an electronic flash unit.

Results

Only the responses of dispersion and aggregation of melanin are dealt with. Experiments were too brief for observable morphological effects, *i.e.* pigment mass change.

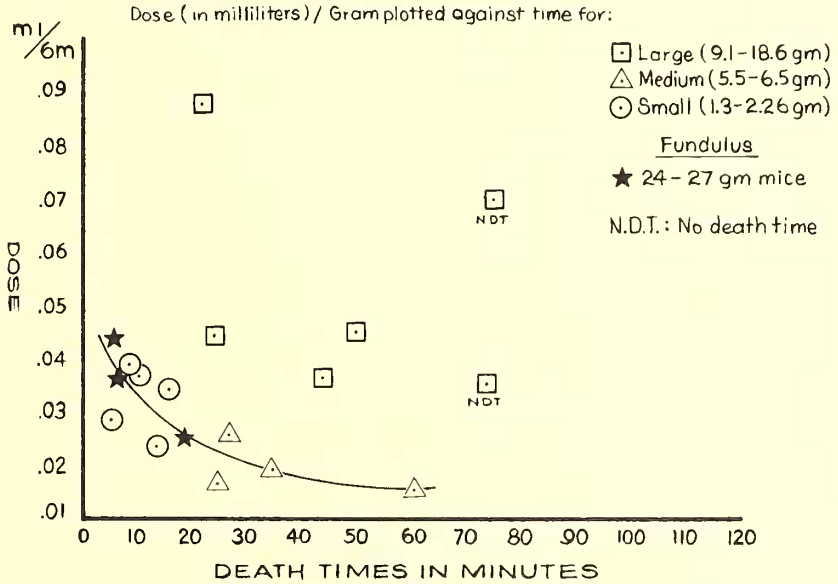
Temperature sensitivity of fish. Local temperature change-induced melanophore effects have been described in *Phoxinus* (Pye, 1964c). We found that transfer of *Fundulus* from seawater at −1.5°C to room temperature or vice versa had no immediate or delayed visible effect except transient equilibrium loss, without visible melanophore reaction.

Hypoxia. Fifty fish held in a water-filled polyethylene bag until hypoxic became dark, and most died. Most survivors regained ability to respond to a white background, but three remained dark. To test melanophore integrity in permanently dark, damaged animals, L-arterenol (norepinephrine) 0.01 mg/g was injected i.p. two days after the hypoxic episode. Within two minutes total blanching occurred, as with normal dark-adapted animals, indicating melanophore integrity of hypoxia-damaged fish.

Size and species differences. We determined the response of various size-ranged animals. *Fundulus* were graded into three size-groups: small (1.3 to 2.2 g.); medium (5.5 to 6.5 g.); and large (9.1 to 18.8 g.). Mice ranged from 24 to 27 g. Saxitoxin was administered i.p. to mice and i.g.b. to fish. Separate tests showed no difference between time or intensity of effects upon i.g.b. versus i.p. injection. Results are shown in chart (Table I). Dosages given to small and medium sized fish and to mice gave death times

Table I

TIME/DOSE RELATIONSHIPS, SAXITOXIN SAMPLE CONTAINING 1.0 MOUSE UNIT/ 0.6 ml (INTRA AIR-SAC INJECTION)



which fell along a single curve. Large fish gave consistent results only with more concentrated solutions, probably because their smaller area of internal absorbing surface per gram of animal and per ml of injected solution vis-a-vis smaller fish resulted in slower toxin absorption. Thus, when animal size is approximately the same, toxin sensitivity of mice is greater than that of *Fundulus*. These results dictated our use of *Fundulus* weighing 7-g or less, to allow use of saxitoxin in the same dilution as for standardization by mouse test. Results were consistent and no correction factor for size was needed.

Peculiarties of Fundulus *vasculature.* We assumed tourniquets might serve to sort out nerve- vs. blood-mediated melanophore responses. Rubber band tourniquets were tried and found ineffective.

Peculiarities of Fundulus *melanophore innervation.*

A. Cholinergic (dispersing) pigmentomotor nerves.

Melanin aggregating nerves of *Fundulus* are known to be adrenergic (Pye, 1964a; Wyman, 1924a). Melanin dispersing nerves when postulated have been assigned cholinergic activity (Pye, 1964c; Fujii and Novales, 1968). We attempted to unmask melanin dispersing nerve action by blocking the sympathetic nervous system with a sympatholytic agent before injecting an acetylcholinesterase inhibitor to increase the concentration of acetylcholine.

Priscoline (tolazoline), an alpha adrenergic blocking agent, upon i.p. injection of 0.02 mg caused a generalized light to dark gray reaction in light adapted animals (4 g) in ~ 10 minutes, with return to normal (light) color at ~ one hour. Eserine (physostigmine), an acetylcholinesterase inhibitor injected in a 2 mg dose in other dark adapted animals caused paling in ~ 10 minutes. This was soon followed by respiratory arrest (cessation of movements of gill operculae) usually preceded by tail spasms and/or convulsions.

Individual drug effects thus established, we gave light adapted *Fundulus* ranging from 1.8-6.8 g priscoline 0.02 mg i.p. and again the partial uniform darkening occurred in ~ 10 minutes. Twenty minutes after i.p. priscoline injection, eserine 2.0 i.g.b. was given each animal. Within 10 minutes respiratory arrest occurred without deepening of the priscoline-induced partial darkening. In a similar experiment light-adapted animals on a light background given doses of priscoline 10x larger (0.2 mg) survived, but melanin dispersal lasted up to five hours. When fish had again become partially light, each was given eserine 2.0 mg and the results were the same, *i.e.*, they progressed to respiratory arrest in ~ 10 minutes without intervening change in degree of melanin dispersion.

B. Adrenergic (aggregating) pigmentomotor nerves.

It has been reported by von Frisch (1911) and widely quoted (Brown, 1957; Pye, 1964a; Wyman, 1924a) that the melanin-aggregating nerves of *Phoxinus phoxinus L.*, a *Fundulus*-like teleost, emerge from the spinal canal at the fifteenth vertebra, bilaterally, and bifurcate anteriad and posteriad (Text-fig. 1).

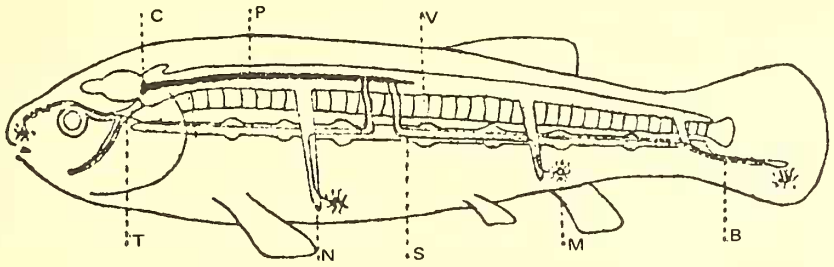
We found the certain minimum lethal dose (M.L.D.) of saxitoxin, given i.p. or i.g.b. to *Fundulus* weighing not over 6 g, to be 0.15 M.U./g (~ 0.03 ug/g.). Half this amount (or more) evokes darkening by quadrants. Usually within one minute, 1-4 sectors (usually right or left anterior) begin to darken (Pl. I, fig. 1). The darkening increases and appears in other sectors (Pl. I, fig. 2), proceeding at an independent rate within each sector until all but the midsection is fully dark (Pl. II, fig. 1). With lethal doses, total body darkening and death ensue (Pl. II, fig. 2). Tetrodotoxin (M.L.D. = 0.075 MU/g = ~ 0.015 ug/g) also causes this sectoral darkening. We believe this illustrates a melanin-aggregating nerve distribution in *Fundulus* similar to that demonstrated in *Phoxinus*.

However, it would be surprising to find that these sympathetic pigmentomotor nerves always emerge from the spinal cord and pass into the sympathetic chain at precisely the fifteenth vertebra. We observed that dark sectors in hundreds of fish, elicited by i.p. or i.g.b. injection of saxitoxin, usually resulted in the pale midzone being centered at or near the fifteenth vertebral level bilaterally. Rarely, the sharply demarcated posterior boundary of one anterior sector and the contralateral sector in a given fish varied in location by as much as four myotomes. Also in some fish, the pale midzone centered several myotomes anteriad, and in others several myotomes posteriad to the fifteenth vertebra. This indicates variability in the level of emergence of the pigment motor nerve fibers.

Discussion

Temperature sensitivity. Even though sudden temperature change had no visible effect upon the shade of intact *Fudulus*, we kept fish at room temperature for 12 hours or longer before using them. This precaution proved worthwhile, for it has since been found (Guttman & Barnhill, 1968) that the excitability of tetrodotoxin-treated axons is more temperature-dependent than that of normal axons.

Hypoxia. That total-animal hypoxia resulted



TEXT-FIG. 1. Diagram of the course of the pigment-motor nerve fibers in the minnow. Pigment-motor centers and fibers in heavy black. Nervous system stippled. (Modified after von Frisch, '11; pl. 4, fig. 6.) *C*, pigment-motor center in medulla; *P*, pigment-motor center in spinal cord; *T*, trigeminal nerve; *N*, spinal nerve; *M*, melanophore; *V*, vertebral column; *S*, sympathetic system with pigment-motor fibers; *B*, spinal nerve to tail.

in complete generalized melanin dispersal contrasts with statements by Wyman (1924a), who cut vessels and nerves, and Spaeth, who used isolated *Fundulus* scales, as related by Wyman (1924a), indicating the effect of anoxia upon *Fundulus* melanophores is aggregation. Although the "anemia contraction" of melanin as observed by Wyman (1924a) could be due to loss of endocrine control, alone or reinforced by loss of oxygen supply, the same reasoning cannot be applied to results with isolated scales as these remain dark in Ringer's solution until oxygen is withdrawn. However, this direct paling effect of anoxia upon melanophores can be explained if melanin dispersal is associated with maintenance of melanophore polarization, and if oxygen is required for this energy-consuming task. Then, if melanophores of hypoxic, intact *Fundulus* still obtain enough oxygen from surrounding water even after depression of the more hypoxia-sensitive nervous system, release from pigment-motor nerve control would allow them to maintain polarity and the accompanying melanin dispersion. Animals remaining permanently dark following an hypoxic episode probably incurred nervous system damage and thus, permanent melanophore release from pigment-motor nerve control—a potentially valuable preparation for other toxin or melanophore research.

Size and species differences. The ratio of internal absorbing surface to animal body mass is important when saxitoxin, tetrodotoxin, and other rapidly absorbable compounds are injected i.p. or i.g.b. Small animals have greater area of visceral plus parietal peritoneal surface per gram than large animals. We observed that saxitoxin m.l.d. per gram for mice (20-plus g) and *Fundulus* in its most abundant sizes (1.5 to 6 g) is nearly the same: the interspecies difference in sensitivity cancels out the difference in sensitivity due to size when animals of these sizes are employed. This is fortuitous, and may or may not hold true for any other given toxin.

Peculiarities of Fundulus *vasculature.* It is probable that our results using tourniquets were negative due to collateral circulation via vessels within vertebral canals. These results supplement fruitless attempts of others (Wyman, 1924a) to sort out endocrine vs. nerve mediated melanophore effects by ligating or otherwise modifying *Fundulus* circulation.

Peculiarities of Fundulus *melanophore innervation.*

A. Cholinergic (dispersing) pigmentomotor nerves.

While direct evidence for melanin-dispersing nerves in *Fundlus* is wanting, many have argued for their existence on the basis of indirect evidence (Fingerman, 1963; von Geili, 1942; Mills, 1932a,b; Parker, 1948; Waring, 1963). Like Scott (1965), we consider the interpretation of the results upon which this indirect evidence is based to be open to question. Electron microscopy has shown but one type of end-plate on *Fundulus* melanophores (Bikle, 1966). Others (Pye, 1964b; Healey, 1954; Healey & Ross, 1966; Abbott, 1968) found no evidence of melanin dispersing nerves in *Phoxinus* or *Fundulus*. Wyman (1924a) concluded that there is no indication of a double innervation to *Fundulus* melanophores. Our results support this view.

Pye (1964c) showed that "regetine appears to suppress all activity in chromatic (paling) nerve tracts while leaving the melanophores free to respond to the humoral influence of large doses of pituitary extracts." He also showed that rogentine does not influence in any way the normal melanin aggregating action of adrenalin directly upon melanophores. Thus, in our attempts to unmask melanin dispersing nerve activity, we propose that the alpha adrenergic blocking agent, priscoline, similar to regetine, causes

melanin dispersal by blocking the melanin aggregating nerves with little if any direct effect on melanophores. If then, fish are made partially dark by priscoline-induced alpha adrenergic nerve blockade, eserine-induced buildup of acetylcholine should cause further melanin dispersal by stimulation only of unblocked cholinergic melanin dispersing nerves, if any exist. But no such effect, nor any other indication of melanin dispersing nerve action, was seen in our priscoline-eserine experiments. We have not yet performed trials substituting beta adrenergic blocking or anti-adrenergic compounds for priscoline.

B. Cholinergic (aggregating) pigmentomotor nerves.

Anatomic features of the pigment-motor nervous system were mentioned under RESULTS (see Text-fig. 1). We believe that darkening by sector, whether induced by saxitoxin or tetrodotoxin, etc., is explainable only on the basis of the anatomic features of the peripheral melanin aggregating nervous system of *Fundulus*. No other known system within the animal (retinal elements, afferent sensory nerves, pigment-motor centers in medulla or spinal cord, etc.) offers an adequate explanation. The elicitation by known neurotoxins supports this interpretation. The midsection nearest the point of nerve emergence from the spinal cord is last to darken, indicating differential nerve sensitivity reminiscent of the ascending paralysis which saxitoxin and tetrodotoxin induce in mammals, including man. Results indicating the probable ionic mechanism for peripheral pigmentomotor system > central nervous system sensitivity to saxitoxin and tetrodotoxin and further confirming specificity of sectoral darkening is given in detail elsewhere (Down & McLaughlin, 1969).

SUMMARY

Using *Fundulus heteroclitus,* a hardy and abundant teleost bait fish in which aggregation-dispersion of melanophore pigment is rapid and predominantly nerve regulated, we found that systemic administration of tetrodotoxin, or saxitoxin cause sectoral darkening, interpreted as following the previously undocumented (in *Fundulus*) pattern of peripheral pigment-motor innervation, indicating peripheral > central nervous system effect, and constituting a sensitive and specific assay system for the toxins. Concurrent quantitative and qualitative analysis of as well as screening for neuroactive toxins is possible using i.p. or i.g.b. *Fundulus* injection.

A combination of our findings and old and new published information leads us strongly to doubt the existence of long-postulated melanin dispersing nerves in *Fundulus heteroclitus.*

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EXPLANATION OF THE PLATES

Effects of saxitoxin (M.L.D.)

Plate I

Fig. 1. Darkening in right anterior sector beginning.

Fig. 2. Both anterior sectors totally or near totally dark.

Plate II

Fig. 1. All four sectors dark, midsection still light, fish listing.

Fig. 2. Postmortem, showing body-wide darkening and muscular atony (held by one gill cover, head down).

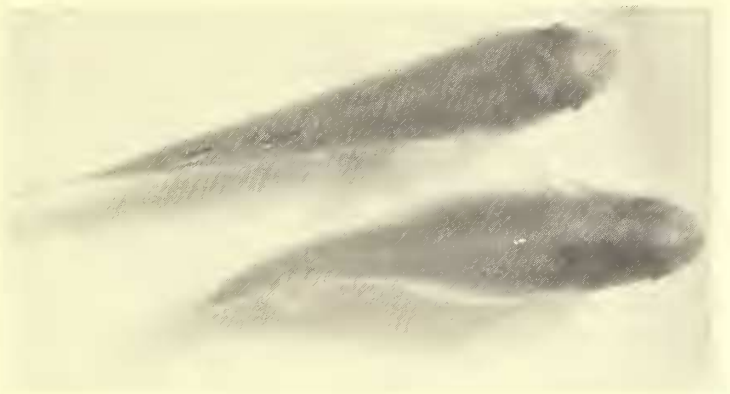

FIG. 1

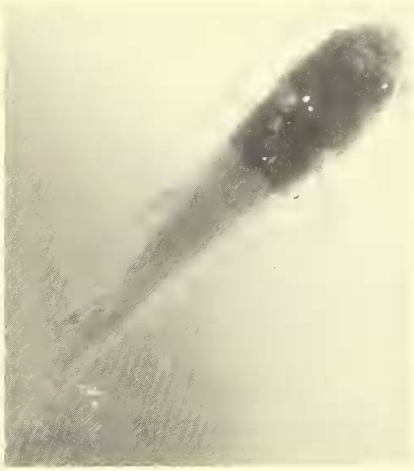

FIG. 2

INTACT KILLIFISH (FUNDULUS HETEROCLITUS) AS A TOOL FOR MEDICALLY ORIENTED STUDY OF MARINE NEUROTOXINS

FIG. 1

FIG. 2

INTACT KILLIFISH (FUNDULUS HETEROCLITUS) AS A TOOL FOR MEDICALLY ORIENTED STUDY OF MARINE NEUROTOXINS